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The Use of 16S rRNA-Targeted Oligonucleotide Probes To Study Competition between Ruminal Fibrolytic Bacteria: Development of Probes for *Ruminococcus* Species and Evidence for Bacteriocin Production

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A total of six oligonucleotide probes, complementary to the 16S rRNA, were evaluated for quantitative and determinative studies of *Ruminococcus albus* and *Ruminococcus flavefaciens*. On the basis of specificity studies, probes for *R. albus* (probe RAL196) and *R. flavefaciens* (probe RFL196) were selected to quantitate these species in mixed culture. In combination with a *Fibrobacter succinogenes* S85 subspecies probe (SUB1) and a domain Bacteria (formerly kingdom Eubacteria) probe (EUB338), they were used to quantitate these species competing in mixed cultures for cellobiose as the carbon source. In dicultures containing *R. albus* 8 and *F. succinogenes* S85, competition was not observed. However, *R. flavefaciens* FD-1 eventually outcompeted *F. succinogenes* S85 when cellobiose was the substrate. When *R. albus* 8 and *R. flavefaciens* FD-1 were grown together on cellobiose medium, *R. albus* 8 outcompeted *R. flavefaciens* FD-1, resulting in undetectable *R. flavefaciens* 16S rRNA only 1 to 3 h after inoculation, suggesting production of an antagonistic compound by *R. albus* 8 during rapid growth on soluble substrates. Further, when *R. albus* 8, *R. flavefaciens* FD-1, and *F. succinogenes* S85 were grown together in a triculture, *R. flavefaciens* FD-1 16S rRNA was detectable for only 2 h after inoculation, while *R. albus* 8 and *F. succinogenes* S85 showed a similar competition pattern to that of the dicultures. The results show that the *Ruminococcus* probes were effective in the measurement of relative populations of selected *R. albus* and *R. flavefaciens* strains during in vitro competition studies with *F. succinogenes*. Moreover, *R. albus* 8 was shown to produce a heat-stable protein factor which causes zones of inhibition in *R. flavefaciens* FD-1 bacterial lawns. This is the first demonstration of the production of a bacteriocin-like substance by a ruminal bacterium. It is postulated that bacteriocin production by ruminal fibrolytic bacteria is a mechanism used to compete for nutrients.

The objectives of this research were to develop molecular methods for analysis of individual population dynamics in mixed cultures of ruminal fibrolytic bacteria. For lack of common analytical tools, the study of microbial population dynamics has been limited. This is changing with increasing application of molecular techniques. In particular, comparative sequencing of the rRNAs offers a powerful and rapid technique for studying the natural relationships of microorganisms (30). Sequence comparisons allow us to infer phylogenetic relationships, and they form the basis for the design of hybridization probes (36). 16S rRNA-targeted synthetic oligonucleotide (17 to 34 nucleotides) hybridization probes have been designed to identify closely related microorganisms (e.g., genus and species) and larger natural assemblages (e.g., domain and family). Such probes have been used for both determinative and quantitative evaluations of environmental populations of microorganisms (1–4, 11, 12, 29–33, 35). Their use does not require cultivation and as such has general utility (16, 18, 31). These studies and others have shown that rRNA-targeted oligonucleotide probes represent a potentially powerful strategy which can be applied in the study of bacterial population dynamics. Furthermore, as the rRNA content of bacteria is proportional to the growth rate (6), relative rRNA levels can be used to estimate the relative activities of different populations.

rRNA-targeted oligonucleotide probes also have enormous potential in the quantitation of bacterial populations in well-defined in vitro mixed-culture experiments. For example, mixed cultures of ruminal fibrolytic bacteria have been used to study various aspects of plant cell wall hydrolysis (10). However, precise measurements of the individual population dynamics in these mixed cultures is mostly lacking as a result of inherent limitations in enumerations dependent on pure-culture isolation of ruminal fibrolytic bacteria. Initial comparative sequencing of the 16S rRNAs of the ruminococci revealed that they constitute a coherent but diverse group of microorganisms (33a) and were amenable to probe development. This work describes the development of 16S rRNA-targeted oligonucleotide hybridization probes for *Ruminococcus albus* and *Ruminococcus flavefaciens* and their application to in vitro competition studies.

(A preliminary report of this work has been presented previously [24b].)

MATERIALS AND METHODS

Bacterial strains. *R. albus* 8, *R. flavefaciens* FD-1, R13e2, B_1C45, $B_1$46, C94, and D101, and *F. succinogenes* S85 were obtained from the culture collection in the Department of Animal Sciences, University of Illinois, Urbana-Champaign. *R.*

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rRNAs. rRNAs from different bacterial strains used to determine the temperature of dissociation (T_d; temperature at which one-half of the hybridized probe remained on the filter after the conditions stated below) and to test the probe specificity were obtained from the stock collection of David Stahl, Department of Veterinary Pathobiology, University of Illinois.

Oligonucleotide synthesis and labeling. Oligonucleotide probes, complementary to regions of the 16S rRNA, were synthesized with a DNA synthesizer (Applied Biosystems, Foster City, Calif.) at the Biotechnology Center, University of Illinois. The 16S rRNA data sequences used were from the data collection of David Stahl, as well as from the Ribosomal Database Project (25). Oligonucleotide probe sites for *R. albus* and *R. flavefaciens* in relation to representative nontarget sequences are shown in Fig. 1. A total of six oligonucleotide probes were designed for the ruminococci, three of which were targeted to sites on the 16S rRNA of *R. albus* and three of which were targeted to sites on the 16S rRNA of *R. flavefaciens*. Table 1 shows the probes that were designed with the numbering based on the corresponding positions in the *Escherichia coli* 16S rRNA. Probe RFL196 was designed to be a strain-specific probe for *R. flavefaciens* FD-1, whereas RFL1176 and RFL1269 were designed to be species-specific probes for *R. flavefaciens*. Probes RAL196, RAL1176, and RAL1269 were designed to be species-specific probes for *R. albus*.

Oligonucleotide probes were labeled at the 5′ end with polynucleotide kinase (Boehringer-Mannheim, Indianapolis, Ind.) and [γ-^{32}P]ATP (ICN Radiochemicals, Costa Mesa, Calif.). The reaction mixture (30 μl) contained 1 μg of oligonucleotide, 3.0 μl of 10× kinase buffer (Boehringer-Mannheim), 1.5 μl of 1% Nonidet P-40, 1.0 μl of polynucleotide kinase (Boehringer-Mannheim), and 1.0 mCi of ATP. The mixture was incubated at 37°C for 30 min. After being labeled, the oligonucleotide probes were purified with Nensorb 20 columns (DuPont NEN, Wilmington, Del.) as specified by the manufacturer.

Extraction of total RNA. rRNA was extracted from 10-ml cultures. Cells were harvested by centrifugation (13,800 × *g*). The cell pellet was taken up in 700 μl of 50 mM sodium acetate–10 mM EDTA (pH 5.1), and zirconium bead disruption was used to isolate total RNA (31). The bacterial cell suspension was placed in a 2.0-ml screw-cap conical tube, and 1.2 g of zirconium beads (diameter, 75 to 200 μm) baked at 300°C overnight, 50 μl of 20% sodium dodecyl sulfate (SDS), and 700 μl of phenol (pH 5.1) were added. The tube was then capped, fitted in a Mini-Bead Beater (Biospec Products, Bartesville, Okla.), and shaken for 2 min. The mixture was then incubated in a water bath at 60°C for 10 min and shaken on the Mini-Bead Beater for an additional 2 min. The mixture was centrifuged (4,000 × *g*) for 3 to 5 min. The aqueous phase was removed and extracted with 500 μl of phenol (pH 5.1). This was followed by at least two extractions with phenol-chloroform and two with chloroform alone. The nucleic acid sample was precipitated overnight at −20°C following the addition of a 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. Following centrifugation and removal of the supernatant, the pellet was washed with 80% ethanol and dissolved in 30 to 50 μl of RNase-free double-distilled H_2O at a concentration of 1 to 2 mg/ml, assuming an optical density at 260 nm of 1 (= 50 μg of RNA per ml). RNA was stored at −20°C.

Oligonucleotide probe hybridization. rRNAs were denatured by adding 3 volumes of 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.0) prior to dilution to 10 ng/100 μl with dilution H_2O containing 50 μg of polyadenylic acid and 1 μl of 2% bromophenol blue. A slot blotter (Schleicher & Schuell, Dassel, West Germany) was used to immobilize rRNA on a MagnaGraph nylon membrane (pore size, 0.45 μm; Micron Separation Inc., Westboro, Mass.). By using a slight vacuum, 10 ng was applied to each slot. The membrane was air dried and then baked at 80°C for 2 h. The membranes were prewetted in hybridization buffer (5 mM sodium phosphate, 0.15 mM EDTA, 0.2 M NaCl, 0.015% SDS, 0.1 mg of polyadenylic acid DNA, 2% Denhardt's solution) (22) and prehybridized for 6 to 8 h. ^{32}P-labeled probes were then added at an activity of 5×10^5 cpm per slot of bound RNA, and the mixture was allowed to hybridize overnight (12 to 16 h) at 40°C. The filters were then washed two or three times in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate)–1% SDS at the empirical wash temperature for each probe.

Quantification of rRNA blots. Hybridized membrane filters were dried and exposed to film (Kodak XRP-5; Eastman Kodak Co., Rochester, N.Y.) with intensifying screens (Cronex Lightning-Plus; E. I. duPont de Nemours and Co., Wilmington, Del.) at −85°C for up to 5 days. After autoradiography, bound probe was quantitated by laser densitometer (Zeineh Soft Laser Scanning Densitometer; Biomed Instruments, Inc., Fullerton, Calif.) as described by Amann et al. (1). A standard curve was generated by using purified 16S rRNA from *E. coli* and dilutions of rRNA of reference bacteria. The domain Bacteria (previously the kingdom Eubacteria) probe (EUB338; 5′-GCT GCC TCC CGT AGG AGT-3′) was used as a positive control (30). The amount of bound probe was converted to micrograms of 16S rRNA to construct a standard curve. The amount of probe bound was also estimated by determining the counts per minute bound to the filters by liquid scintillation counting. The standard curve was used to estimate the actual amount of 16S rRNA in the samples. It should be noted here that when used to quantitate microorganisms, the specific/total rRNA ratio is only an approximate estimate of relative cell numbers, since cellular rRNA content varies with growth rate. All the experiments were repeated two or three times, and the mean amount of 16S rRNA (in micrograms) was used. These data were reproducible under the conditions used.

Hybridization to RNA from cellobiose-grown cultures. Complex medium (24) with 0.4% cellobiose was used in all experiments. Balch bottles containing 200 ml of anaerobically prepared medium (7) were inoculated with 10 ml of an exponential-phase cellobiose-grown culture (optical density at 600 nm, approximately 0.6). The optical density at 600 nm of the culture supernatant was recorded for each culture to monitor growth. Cultures were grown without agitation and were mixed by inversion prior to each sampling. Samples (10 ml) were collected every 1 h for total RNA extraction (see above). The following probes were used: RAL196 (Table 1), RFL196 (Table 1), *F. succinogenes* subsp. *succinogenes* probe SUB1 (31), and the domain Bacteria probe (EUB338) (30). The abundance of organisms was expressed as a percentage of the total 16S rRNA in the sample (relative RNA index [23]). For protein determinations, the Coomassie blue dye-binding method was used with bovine serum albumin as the standard (5).

Substrate disappearance. The phenol-sulfuric acid procedure (13) was used to determine substrate disappearance from cellobiose-grown cultures.

Bacteriocin screening. The procedures of Geis et al. (14) were used to sceen for antagonistic activities. Overnight cultures of cellobiose-grown *R. albus* 8 were spotted on cellobiose

PROBE SITES FROM POSITION 196 TO 213

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60%consen     130  -GAAA-+GA++++UAAUACCGCAUAA+-AU++++++-----+UC-GCAU-G+U
Rum100%       159  -GAAA-CG++UGCUAAUaCCGCAUAAC-AUAU+U+A ----+CC-GCAU-GGC
R. albus probe site                            -AUAACGAA-----GCC-GCAU-GAC
R. albus8      17  -gaAA-CGAUUGUUAAUaCCUCAUAAC-AUAACGAA-----GCC-GCAU-GAC
R. albus7       0                               UAACGAA-----GCC-GCAU-GAC
R. bromJ32A    10  -GAAA-AGAAUGCUAAUACCGCAUGAy-AUAUCGGA-----ACC-ACAU-NGU
R. brom6833    14  -GAAA-AGAAUGCUAAUaCCGCAUGAC-AUAUCGGA-----ACC-ACAU-NGU
br56B47         0    -aa-agaaugcuaauaccgcAUAAC-AUAUAUUU-----AUC-GCAU-GGU
RcHS7A4         0
R. albusKB1     0                  -CCNcauaaC-AUAGAAAA-----GCC-GCAU-NaC
R.flav probe site                              AUAAUUGA-----AGG-GCAU-CCU
R. flvC94      13  -gaaa-cggaugGUAAUaCCUCAUAaC-AUAACUGA-----ACC-GCAU-gAU
R. flvFD1     156  -GAAA-CGGAUGGUAAUaCCUCAURAC-AUAAUUGA-----AGG-GCAU-CCU
E. coli       169  -GAAA-CGGCUGCUAACAccGCAUaAG-CUGUACAA-----UAC-GCAU-GUU
C. bryantii   163  -GAAA-CGACUgCUgAUACCGCAUaAG-CUUCC--------UgA-UUaA-UUG
C. pasteur    148  -GAAA-GGGaGAUUAAUACCGCAUAAU-AUUAAAGU-----UUC-ACAU-GGA
C. botulin    151  -GAAA-GGgaGaUUaAUaCCgcaUaAA-GUAUGAAG-----GUC-gcaU-GAc
C. kluyver    153  -GAAA-GGGAGAUUAAUACCGCAUAGA-AGGUAAAA-----AUC-GCAU-GAU
C. cochlear   156  -GAAA-GGAGGAUUAAUACCGCaUaAA-GUUAGAGU-----UUC-GCAU-GAA
C. limosum    155  -GAAA-GGAAGAUUAAUACCGCaUaAu-AUCAUUUu-----AAU-GCaU-GUU
C. novii      157  -GAaa-GGAAGAUUAAUACCGCAUAAU-AUGAGAGa-----AUC-GCAU-gAU
C. ocean      155  -GAAA-GGAGgaUUAAUACCGCAUAAC-GUAAGAGU-----AUC-GCAU-gGU
C. carnis     156  -gAAA-GGAAGAUUAAUACCGCAUAAU-AUUGCagc-----uUC-GCAU-GAA
C. perfrin    155  -GAAA-GGAAGAUUAAUACCGCAUAAu-GUUGAAAG-----AUG-GCAU-CAU
C. symbios    171  -GAAA-UGACUGCUAAUACcgCAUAAG-CGCACAGU---- AUu-GCAU-GAU
C. oroti      175  -GAAA-UGACUGCUAAUACCGUAUAAG-AccACAgu-----gcC-GCAU-GGC
C. cocco      171  -GAAA-UGACUGCUAAUACCGCnUnAG-CGCACAGG-----ACC-GnnU-GGU
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PROBE SITES FROM POSITION 1176 TO 1193

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60%consen     963  +U+AG+-------+UG+GCACUCUAG+GAGACUGCC+++--GA+AA--+++G-GAGGAA
Rum100%       938  GCAAG----------AGCACUCUA+++GGACUGCCGUU--GACaA--AACG-GAGGAA
R. albus probe site                      CUAGCAGGACUGCCGUU
R. albus8     965  GCAAG----------AGCACUCUAGCAGGACUGCCGUU--GACAA--AACG-GAGGAA
R. albus7     925  GCAAG----------AGCACUCUAGCAGGACUGCCGUU--GACAA--AACG-GAGGAA
R. albusKB1   933  GCAAG----------AGCACUCUAGCAGGACUGCCGUU--GACAA--AACG-GAGGAA
R. bromJ32A   963  GCAAG----------AGCACUCUAAUAGGACUGCCGUU--GACNA--AACG-GAGGAA
R. brom6833   965  GCAAG----------AGCACUCUAAUAGGACUGCCGUU--GACNA--AACG-GAGGAA
brS6B47       950  GCAAG----------AGCACUCUAAUAGGACUGCCGUU--GACNA--AACG-GAGGAA
RcHS7A4       532  GCAAG----------AGCACUCUAAUAGGACUGCCGUU--GACAA--AACG-GAGGAA
R.flav probe site                      CUAAAGGGACUGCCGUU
R. flvC94     973  GCAAG----------AGCACUCUAAAGGGACUGCCGUU--GACAA--AACG-GAGGAA
R. flvFD1    1106  GCAAG----------AGCACUCUAAAGGGACUGCCGUU--GACAA--AACG-GAGGAA
E. coli      1165  UaAUG-------AUGAGCaCUCUAAUGGGACUGCCUGU--GAcaA--GCaG-GAGGAA
S. bryantii  1153  AUAAGG-------UGAGCaCUCUaAUgGGACuGCCUGU--GACAA--GCAG-GAGGaA
C. pasteur   1103  UUAAGU-------UGAGCACUCUAGUGAGACUGCcCGG--GUUAA--CCGG-naGGAA
C. botulin   1105  UUAAGU-------UGAGCACUCUAGUGAGACUGCCcGG--GuunA--CCGG-GAGGAA
C. kluyver   1108  GUAAAG------GUGGGCaCCCUAACGaGACUGCcGCg--GcuAA--CGUG-GAGGAA
C. cochlear  1110  UUAAGU-------UGAGCACUCUAAUGAGACUGnccGG--GUgAA--CCGG-gaGGAA
C. limosum   1111  UUAAGU-------UGAGCACUCUAGCAAGACUGCCUGG--G-Uaa--CCaG-GAGGAA
C. novii     1110  UUAAGU-------UGAGCACUCUAAUGAGACUGCCUGG--G-UaA--CcaG-GAGGAa
C. ocean     1107  UUAAGU-------UGAGCACUCUAAUGAGACUGCCUGG--G-UAA--CCAG-GaGGAA
C. carnis    1111  UUUAGU-------UGAGCAcuCUAGCGAGACUGcccGG--GUUaA--CcGG-gaGGAA
C. perfrin   1110  UUAAGU-------UGAGGACUCUAGCGaGACUGCCUGG--GuuAA--CCAG.GaGGAA
C. symbios   1125  UUCGG-------CCGGgaACUCUUGGgaGACUGCCAgG--gAUAA--CCUG-GAGGAA
C. oroti     1129  cUuGG-------CCGGGCACUCUAGAGAGACUGCcAGG--GAUAA--CCUG-GAGGAA
C. cocco     1125  AUGaUG------GUGGGCaCUCUAGGGaGACUGCCGGG--GAUnA--CCCG-GAGGAA
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PROBE SITES FROM POSITION 1269 TO 1280

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60%consen     1014  GCUACACACGUGCUACAAUGG+++++A-C++AG+G+AGC+A++C+-G+GA-GGUGGAG
Rum100%       1018  GCUACACACGUACUAC+AUGG+++UUAAC++AG+G+AGCAA++C+-G+GA--+G++GAG
R. albus probe site                    AUGGCUGUUAACRGAGGGAA
R. albus8     1049  GCUACaCACGUACUACNAUGGCUGUuAAcaGAGGGAAGCAAAGCA-GYGA-UGCAGAG
R. albus7     1009  GCUACACACGUACUACrAUGGCUGUUAACNGAGGGAAGCAAAACA-GUGA-UGUGGAG
R. albusKB1   1017  GCUACACACGUACUACmAUGGCUGUUAACaGAGGGAAGCAAAACA-GUGA-UGUGGAG
R. bromJ32A   1047  GCUACACACGUACUACNAUGGACAUUAACRGAGGGAAGCAAUACA-GCGA-UGUGGAG
R. brom6833   1049  GCUACNCaCGUACUACNAUGGAUGUUAACvGAGGGAAGCAAGACA-GUGA-UGUGGAG
br56B47       1034  GCUACaCaCGUACUACNAUGGAUGUUAACRGAGGGAAGCAAGACA-GCGA-UGUGGAG
RcHS7A4        616  GCUACACACGUACUACNAUGGCAAUUAACNAAGAGCAGCAAAGcV-GCGA-GGUGGAG
R. flav probe site                     AUGGCAAUUAACAAAGAGAA
R. flvC94     1047  GCUACACACGUACUACaAUGGCAAUUAACaAAGAGAAGCAAGACG-GUGA-CGUGGAG
R. flvFD1     1190  GCUACACACGUACUACaAUGGCAAUUAACAAAGAGAAGCAAGACA-GCGA-UGUGGAG
E. coli       1252  GCUACaCaCGUGCUaCnAUGGAUGGUACaagAGGGcaGUGAAGCc-GUGA-GGUGAAG
S. bryantii   1240  GCUACACACGUGCUaCaAUGGAUGGUA-CAGAGAGCAGCGAGGCC-GCGA-GGUGGAG
C. pasteur    1190  GCUACACACGUGCUACAAUGGUGAGAA-CAACGAGAUGCAAUACC-GUGA-GGUGGAG
C. botulin    1192  GCUACACACGUGCUACAAUGGUUGGUA-CAACaAGAUGCAAGACC-GCGA-GGUGGAG
C. kluyver    1196  GCAACACACGUGCUACAAUGGGCAGAA-CAGAGAGAAGCAAUnCC-GCGA-GGAGGAG
C. cochlear   1197  GCUaCaCACGUGCUACnAUGGUGGGUA-CaGAGAGAAgcgAUACC-GCGA-GGUGGAG
C. limosum    1197  GCUaCaCaCGUGCUACAAUGGUUGGUA-CAAAGAGAAGCAAGACC-GUGA-GGUGGAG
C. novii      1196  GCUACACACGUGCUACAAUGGUAGGUA-CAAUAAGACGCAAGACC-GUGA-GGUGGAG
C. ocean      1193  gCUACACACGUGCUACAAUGACAGGUA-CAGAGAGACGCAAGACC-GUGA-GGUGGAG
C. carnis     1198  GCUaCaCaCGUGCUACAAUGGCAAGUA-CaAAGAGAUGCAAUACC-GCGA-GGUGGAG
C. perfrin    1197  gCUACACACGUGCUACAAUGGCUGGUA-CAGAGAGAUGCAAUACC-GCGA-GGUGGAG
C. symbios    1212  gCUACACACGUGCUaCAAUGGCGUAAA-CanAGAGAAGCAAgACC-GCGA-gGUGGAG
C. oroti      1216  GCUACACACGUGCUACAAUGGCGUAAA-CAAAGGGAGGCAAUACU-GUGA-AGUGGAG
C. cocco      1213  GCUACACACGUGCUACaAUGGCGUAAA-CnAAGGGAAGCGAGACA-GCGA-UGUUGAG
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FIG. 1. Oligonucleotide probe target sites for *R. albus* and *R. flavefaciens* in relation to representative nontarget sequences. The sequences are shown 5′ to 3′. Capital letters denote bases assigned with certainty; lowercase letters denote bases assigned with ambiguity; *n* denotes an ambiguous band on a sequencing gel without assignment of base; + denotes deviation below the consensus sequence; and − denotes a gap where no consensus nucleotide is present at that position when aligned against *E. coli* 16S rRNA sequence. The target regions for the six probes designed are underlined.

TABLE 1. Probes designed for *R. albus* and *R. flavefaciens*

Probe	Position[a]	l[b]	Sequence	T_d (°C)	
				Empirical	Estimated[b]
RAL196	196–213	18	5′-GTC ATG CGG CTT CGT TAT-3′	46	45
RFL196	196–213	18	5′-AGG ATG CCC TTC AAT TAT-3′	45	40
RAL1176	1176–1193	17	5′-AAC GGC AGT CCT GCT AG-3′	44	45
RFL1176	1176–1193	17	5′-AAC GGC AGT CCC TTT AG-3′	46	43
RAL1269	1269–1280	17	5′-TTC CCT CTG TTA ACA GCC AT-3′	46	47
RFL1269	1269–1280	17	5′-TTC TCT TTG TTA ATT GCC AT-3′	45	41

[a] *E. coli* numbering.
[b] T_d estimated by using the equation of Lathe (20) $T_d = 81.5 + 16.6 \log M + 0.41(\%\ G + C) - 820/l$, where M is the monovalent cation concentration and l is the length of the probe in nucleotides.

agar plates (complex medium with 0.4% cellobiose) and incubated at 39°C for 18 h. About 3.5 ml of 0.7% agar (complex medium with 0.4% cellobiose) was inoculated with a late-exponential-phase culture of *R. flavefaciens* FD-1 (optical density at 600 nm, 2.0), poured onto the surface of the agar plates, and incubated at 39°C. The plates were examined for zones of inhibition after 6, 12, and 24 h.

Partial purification of bacteriocin-like substance. *R. albus* 8 was grown overnight in 250 ml of complex medium with cellobiose as the carbon source. The procedures used for bacteriocin extraction were those of Daba et al. (8). The overnight culture was centrifuged at 13,800 × *g* for 10 min. The supernatant was saturated to 60 and 80% with ammonium sulfate and stirred at 4°C overnight. The sample was centrifuged at 13,800 × *g* for 20 min, and the pellet was taken up in 5 to 10 ml of 0.1 M phosphate buffer (pH 6.0) and dialyzed (1.4-kDa cutoff) against 1.0 liter of 0.1 M potassium phosphate buffer (pH 6.0) overnight. Part of the solution remaining in the dialysis tube was then sterilized by filtering through a 0.22-μm-pore-size filter (Costar, Cambridge, Mass.). The cell-free supernatant then was tested for bacteriocin activity against *R. flavefaciens* FD-1. An overnight culture of cellobiose-grown *R. flavefaciens* FD-1 was spread uniformly over a cellobiose agar plate with a glass rod. Wells were bored through the agar by using the wide part of a sterile Pasteur pipette. The wells were filled with about 100 μl of the potential bacteriocin solution and then incubated at 39°C for 12 to 48 h.

Effect of proteolytic enzymes on bacteriocin-like substance. The effect of proteolytic enzymes on the potential bacteriocin was tested with pronase E and α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) by the procedures of Davey and Richardson (9). The two enzymes were dissolved in 0.01 M phosphate buffer (pH 7.0) to give a solution of 0.1 mg of the enzyme per ml. The partially purified bacteriocin sample was mixed with an equal volume of the enzyme (200 μl), and the mixture was incubated at 37°C for 1 h. Bacteriocin samples without added enzyme served as the control and were treated in the same manner. The samples were then boiled for 10 min to inactivate the proteins and assayed for bacteriocin activity.

RESULTS

Probe design and characterization. Six potential *Ruminococcus* probes were designed following sequence alignment and inspection (Table 1). The specificity of the six *Ruminococcus* probes complementary to 16S rRNAs of different ruminal and nonruminal bacteria is shown in Fig. 2. The *R. albus* probes RAL196 and RAL1176 hybridized to rRNA from *R. albus* 8 but not to any other organism examined. Probe RAL1269 also hybridized with rRNA from *R. bromii* 6883 and therefore was not considered specific for *R. albus* strains. The *R. flavefaciens* probe RFL196 was designed to be strain specific for *R. flavefaciens* FD-1. However, this probe also hybridized with *R. flavefaciens* R13e2 (Fig. 2) and thus cannot be considered strain specific for *R. flavefaciens* FD-1. *R. flavefaciens* probe RFL1176 bound to rRNA from all the strains of *R. flavefaciens* tested but also hybridized with rRNA from *Eubacterium aerofaciens* (Fig. 2). This probe may be useful in identifying and quantifying *R. flavefaciens* strains in an environment where *E. aerofaciens* is absent. *R. flavefaciens* probe RFL1269 hybridized to most *R. flavefaciens* strains but gave weak signals with *R. flavefaciens* strains B34b and $B_1$46.

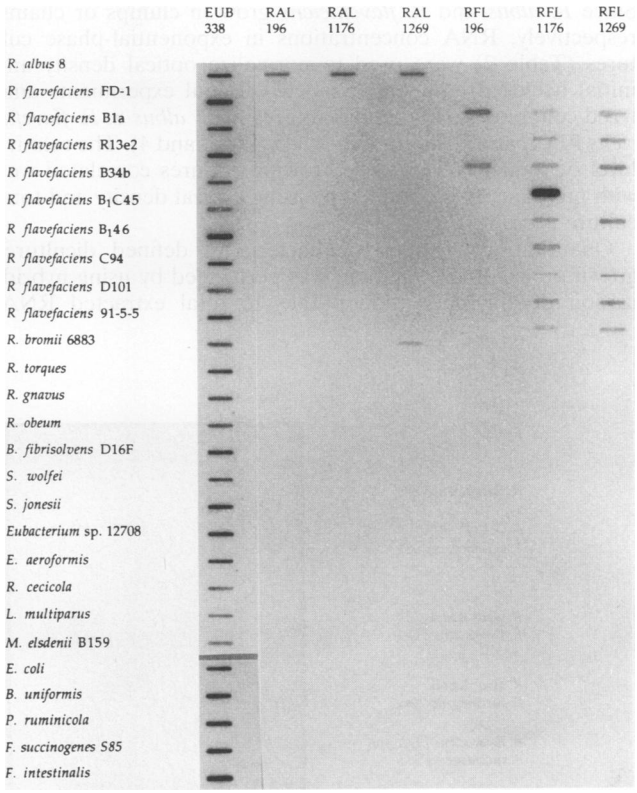

FIG. 2. Species and strain specificity of the probes designed. Approximately 20 ng of rRNA was blotted in each slot blot. The domain Bacteria probe, EUB338, was used as a positive control. The wash temperatures are shown in Table 1. The film was exposed for 3 days. Abbreviations: EUB338, domain Bacteria probe; RAL196, *R. albus* probe 1; RAL1176, *R. albus* probe 2; RAL1269, *R. albus* probe 3; RFL196, *R. flavefaciens* probe 1; RFL1176, *R. flavefaciens* probe 2; RFL1269, *R. flavefaciens* probe 3.

TABLE 2. Bacterial cell numbers and total RNA content from cellobiose-grown inocula

Bacterium[a]	10^{10} No. of cells	RNA content ($\mu g/10^7$ cells)
R. albus 8	5.24	0.18
R. flavefaciens FD-1	5.0	0.15
F. succinogenes S85	6.7	0.16

[a] Optical density, 0.6.

The T_d corresponding to the wash temperatures for each probe was determined experimentally as well as by using calculations of Lathe (20) ($T_d = 81.5 + 16.6 \log M + 0.41[\%G + C] - 820/l$, where M is the monovalent cation concentration and l is the length of the probe in nucleotides). Table 1 shows the empirically determined and estimated (20) values. The wash temperatures for all the probes were comparable (Table 1). This was not surprising, because the probes have the same target site on the rRNA molecule. The empirically determined T_d was used as the final wash temperature in the subsequent hybridization studies.

Quantitation of fibrolytic bacteria in cellobiose-grown cultures. From the specificity studies, probe RAL196 was chosen for quantitation of *R. albus* 8 and probe RFL196 was chosen for quantitation of *R. flavefaciens* FD-1 in pure-culture competition studies with cellobiose as the substrate. *F. succinogenes* S85 was quantified by using the 16S rRNA probe SUB1 (31). Since *R. albus* and *R. flavefaciens* grow in clumps or chains, respectively, RNA concentrations in exponential-phase cultures (Table 2) were used to normalize optical density and initial bacterial numbers in inocula. Initial experiments analyzed cellobiose-grown monocultures of *R. albus* 8, *R. flavefaciens* FD-1, and *F. succinogenes* S85 (Fig. 3 and 4). The relative level of total RNA in these growing cultures correlated well with growth curves obtained by using optical density and total culture protein (Fig. 4).

Quantitation of fibrolytic bacteria in defined dicultures grown on cellobiose medium was performed by using hybridization of oligonucleotide probes to total extracted RNA. When *R. albus* 8 and *R. flavefaciens* FD-1 were grown together on cellobiose medium, *R. albus* 8 outcompeted *R. flavefaciens* FD-1 (Fig. 3 and 5). *R. flavefaciens* FD-1 was present in the culture for only 1 to 3 h after inoculation, suggesting that after 1 h of incubation, the population of *R. flavefaciens* FD-1 in the culture started to decrease while that of *R. albus* 8 increased and accounted for most of the cultures total RNA. In dicultures containing *R. albus* 8 and *F. succinogenes* S85 the relative proportions of the two bacteria were similar but there seemed to be slightly more *F. succinogenes* S85 than *R. albus* 8 once the substrate was depleted (Fig. 3 and 6). These results suggest that after the cellobiose has been exhausted (4 to 6 h), *F. succinogenes* S85 may have better survival mechanisms than *R. albus* 8. In contrast, in dicultures containing *R. flavefaciens* FD-1 and *F. succinogenes* S85, *R. flavefaciens* FD-1 appears to slowly outcompete *F. succinogenes* S85 (Fig. 3 and 7). Finally, when *R. albus* 8, *R. flavefaciens* FD-1, and *F. succinogenes* S85 were grown together in triculture (Fig. 3 and 8), *R. flavefaciens* FD-1 16S rRNA was detectable for only 2 h after inoculation, while *R. albus* 8 and *F. succinogenes* S85 showed similar proportions to those in the diculture experiment (Fig. 3 and 6).

Identification of a bacteriocin-like substance. The above results suggest that *R. albus* 8 produced a substance(s) that inhibits the growth of *R. flavefaciens* FD-1 but not *F. succinogenes* S85. Therefore, we tested the hypothesis that *R. albus* 8 was producing a bacteriocin-like substance that was inhibiting the growth of *R. flavefaciens* FD-1. Overnight cultures of cellobiose-grown *R. albus* 8 were spotted on cellobiose agar plates, and then a 0.7% agar overlay containing *R. flavefaciens* FD-1 was applied. Colonies of *R. albus* 8 had well-defined zones of inhibition around them (data not shown). All the colonies had large and well-defined zones around them after 12 h, whereas at 24 h the zones were large and covered almost the entire surface of the plate. Similar experiments with *F. succinogenes* S85 as the indicator strain showed no zone of inhibition resulting from *R. albus* 8 colonies (data not shown). These data clearly demonstrate that *R. albus* 8 was producing an antagonistic substance(s) capable of inhibiting the growth of *R. flavefaciens* FD-1 but not *F. succinogenes* S85.

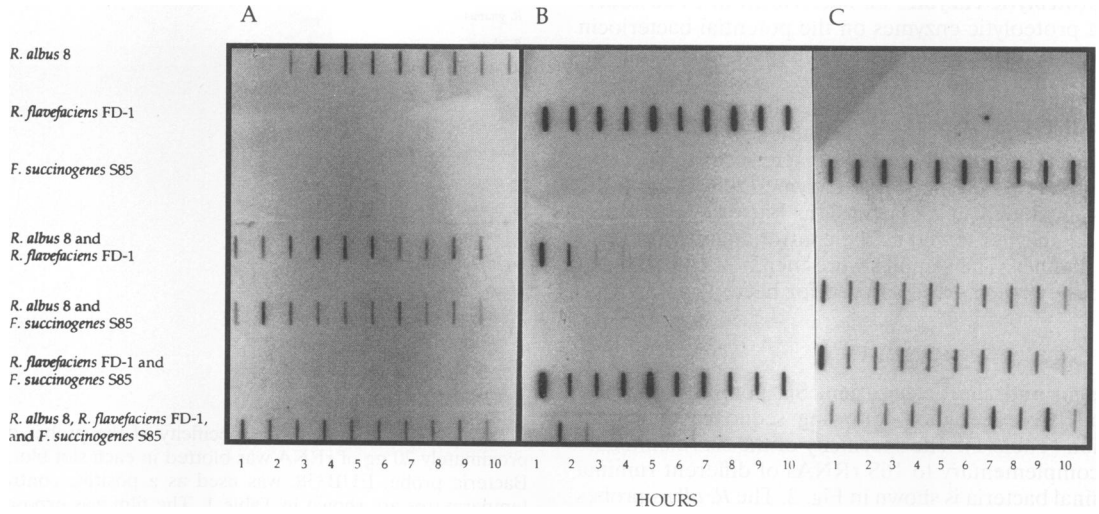

FIG. 3. Slot blots of total RNA from cellobiose-grown monocultures, dicultures, and triculture of *R. albus* 8, *R. flavefaciens* FD-1, and *F. succinogenes* S85. Total RNA was extracted by the bead disruption method. Approximately 40 ng of total RNA was blotted per slot. The film was exposed for 3 days. (A) RNA probed with the *R. albus* probe RAL196. (B) RNA probed with the *R. flavefaciens* probe RFL196. (C) RNA probed with the *F. succinogenes* probe SUB1.

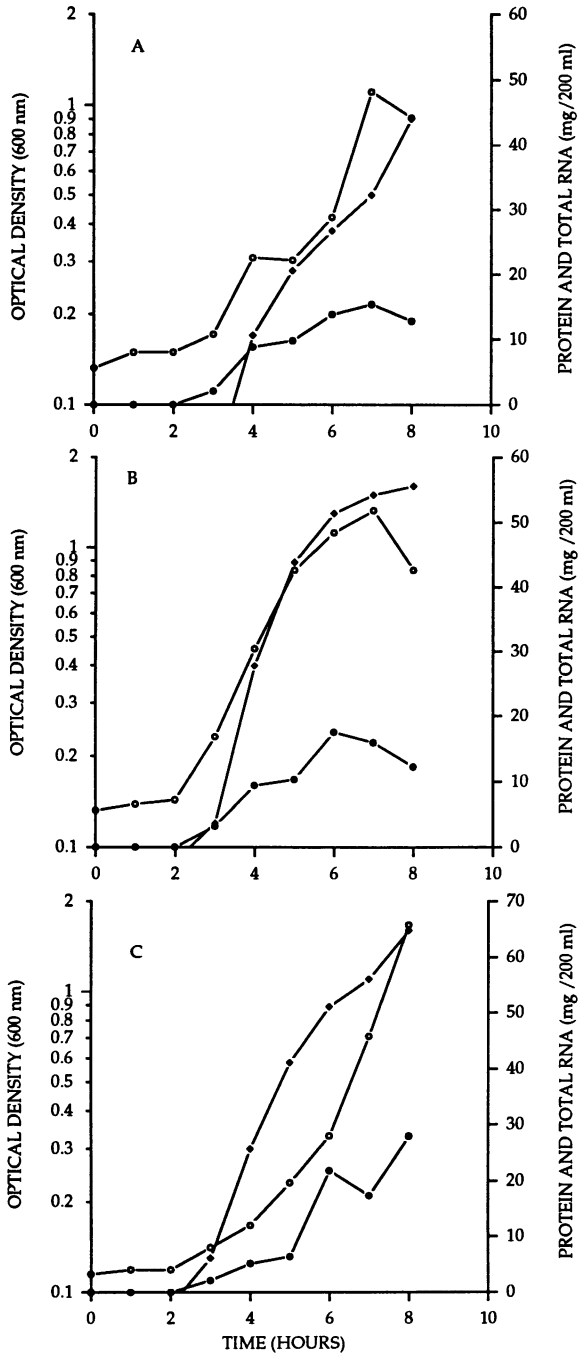

FIG. 4. Growth of cellobiose monocultures of *R. albus* 8 (A), *R. flavefaciens* FD-1 (B), and *F. succinogenes* S85 (C) measured by observing the optical density (◆), total culture protein concentration (○), and total RNA concentration (●).

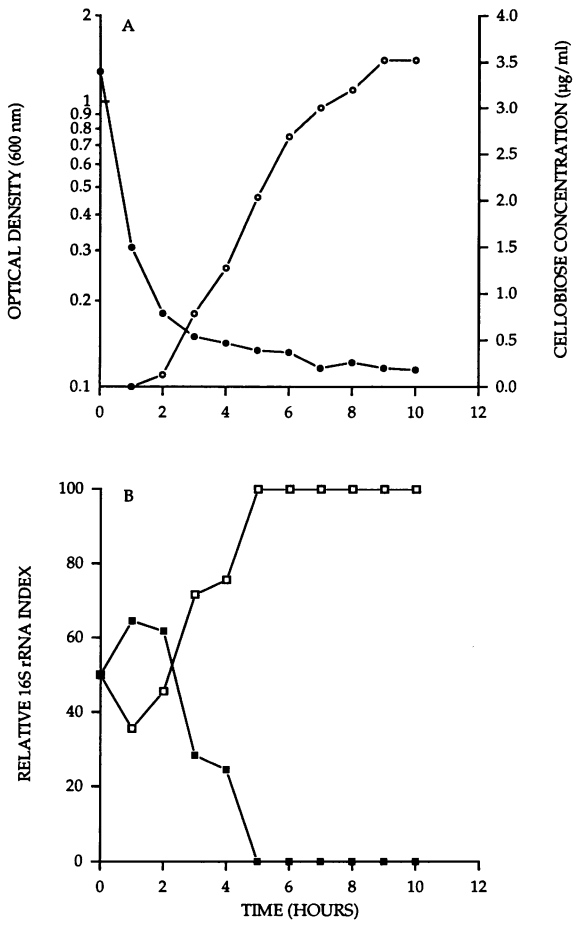

FIG. 5. Bacterial growth, substrate disappearance, and relative 16S rRNA indexes (percent) of a cellobiose-grown diculture of *R. albus* 8 and *R. flavefaciens* FD-1. (A) Bacterial growth (○) measured by observing the optical density at 600 nm, and substrate disappearance (●) measured as the total sugar concentration. (B) Slot blot quantitation of *R. albus* 8 (□) and *R. flavefaciens* FD-1 (■) with *R. albus* probe RAL196 and *R. flavefaciens* probe RFL196, normalized to EUB338.

The cell-free culture supernatant from *R. albus* 8 was then tested for bacteriocin-like activity against *R. flavefaciens* FD-1 by using agar plates with wells which contained cell-free culture supernatants from *R. albus* 8. Zones of inhibition were clearly detectable by this technique. The bacteriocin-like substance in the cell-free culture supernatant from *R. albus* 8 was further characterized by partial purification via ammonium sulfate precipitation. The 60% ammonium sulfate-saturated supernatant sample contained the majority of the bacteriocin-like activity. Following dialysis, both nonfiltered and filtered (pore size, 0.22 μm) supernatant samples had antagonistic activity (Fig. 9). However, the unfiltered sample showed more prominent zones of inhibition (Fig. 9, well D) than the filtered sample did (Fig. 9, wells B and C). Treatment with both pronase E and α-chymotrypsin abolished the antagonistic activity, whereas boiling for 10 min did not (data not shown). These results suggest that the substance is peptide in nature and may be a bacteriocin. Further investigations are, however, needed to examine the host range and confirm that the substance produced by *R. albus* 8 against *R. flavefaciens* FD-1 is a true bacteriocin.

DISCUSSION

The above studies indicate that oligonucleotide probes designed from 16S rRNA sequences can be used to quantify fibrolytic bacteria in defined mixed cultures. The hybridization probes designed in this study provide a highly sensitive means for rapid detection and quantification of *R. albus* and *R.*

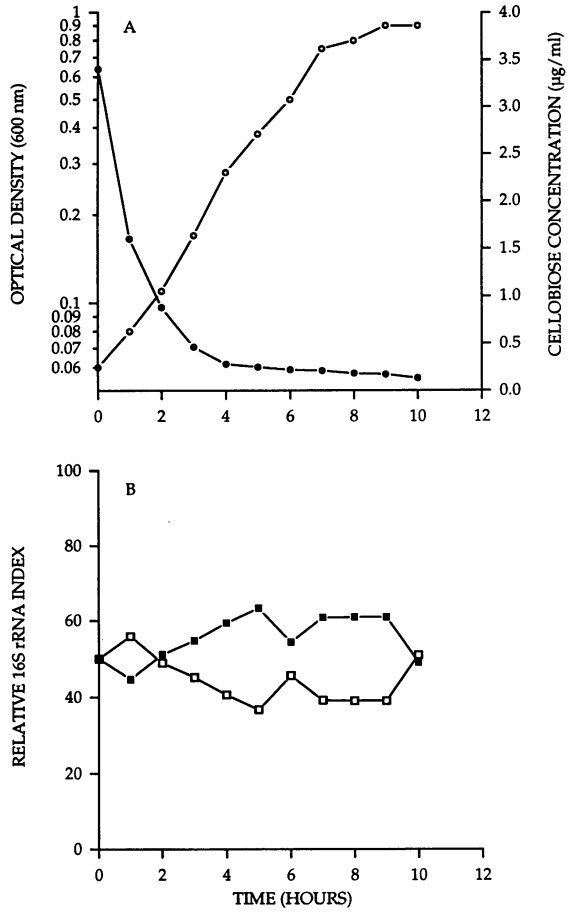

FIG. 6. Bacterial growth, substrate disappearance, and relative 16S rRNA indexes (percent) of a cellobiose-grown diculture of *R. albus* 8 and *F. succinogenes* S85. (A) Bacterial growth (○) measured by observing the optical density at 600 nm, and substrate disappearance (●) measured as the total sugar concentration. (B) Slot blot quantitation of *R. albus* 8 (□) and *F. succinogenes* S85 (■) with *R. albus* probe RAL196 and *F. succinogenes* probe SUB1.

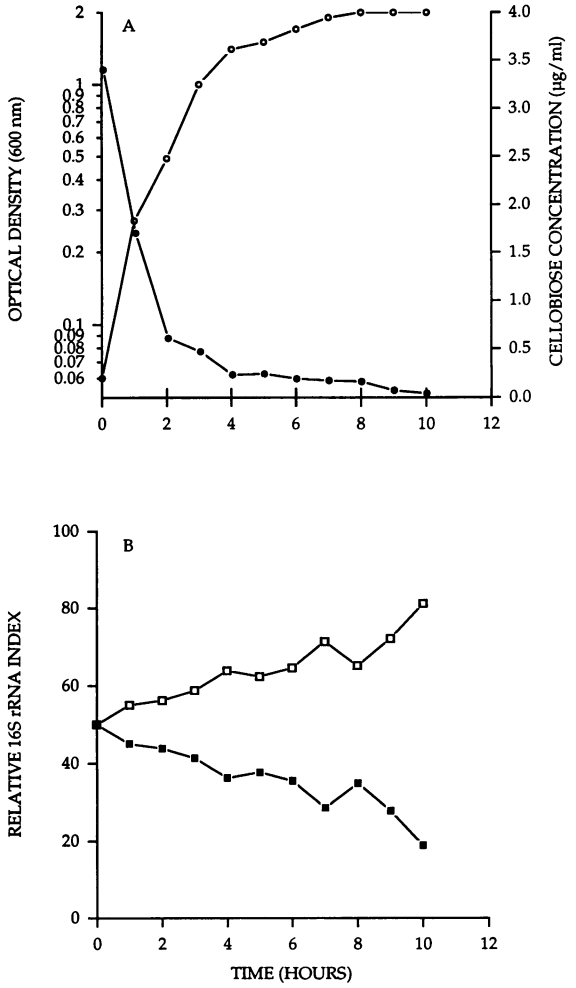

FIG. 7. Bacterial growth, substrate disappearance, and relative 16S rRNA indexes (percent) of a cellobiose-grown diculture of *R. flavefaciens* FD-1 and *F. succinogenes* S85. (A) Bacterial growth (○) measured by observing the optical density at 600 nm, and substrate disappearance (●) measured as the total sugar concentration. (B) Slot blot quantitation of *R. flavefaciens* FD-1 (□) and *F. succinogenes* S85 (■) with *R. flavefaciens* probe RFL196 and *F. succinogenes* probe SUB1.

flavefaciens strains in vitro in experiments with defined ruminal organisms. Further, if species-specific probes have been designed for the other organisms in the defined mixed cultures, the population dynamics for all members of the culture can be determined. In this case, relative levels of *F. succinogenes* were also measured. This is in sharp contrast to previous studies with mixed cultures of ruminal fibrolytic bacteria. These studies have been used to study aspects of plant cell wall hydrolysis such as substrate disappearance rates, residual monosaccharide contents of plant material, substrate preferences, and synergistic effects on degradation (10). However, these studies are lacking in that the actual population levels and kinetics of growth of the individual bacteria in these defined mixed cultures were not characterized. The techniques reported in this study allow rapid and accurate measurement of the population dynamics in mixed cultures containing the major ruminal fibrolytic bacteria.

The competition between these three ruminal fibrolytic bacteria when grown on cellobiose as the carbon source, as demonstrated by relative 16S rRNA levels, illustrates some interesting aspects that have not been previously described. Remarkably, competition between these fibrolytic bacteria was not detected in previous studies involving other measurements of bacterial interactions (24). Nonetheless, it is clear that under the conditions of these studies there is little competition between *F. succinogenes* S85 and *R. albus* 8 when cellobiose is the carbon source. If there is any competition between these two bacteria, it is manifested when substrate is limiting. It is likely that a major component of bacterial competition in the rumen also occurs when carbon and energy are limiting. This nutritional competition is also evident in the cocultures containing *F. succinogenes* S85 and *R. flavefaciens* FD-1. The slow but steady advantage of *R. flavefaciens* FD-1 relative to *F. succinogenes* S85 could reflect a nutritional advantage inherent in *R. flavefaciens* FD-1 (μ_{max} or K_m) such as the rate of substrate uptake (V_{max}) or in the coupling of energy to growth related processes. Cellobiose uptake has been measured in both of these bacterial strains (17, 21), and the results would suggest that the rates of cellobiose transport do not confer upon *R. flavefaciens* FD-1 a competitive advantage. Rather, the

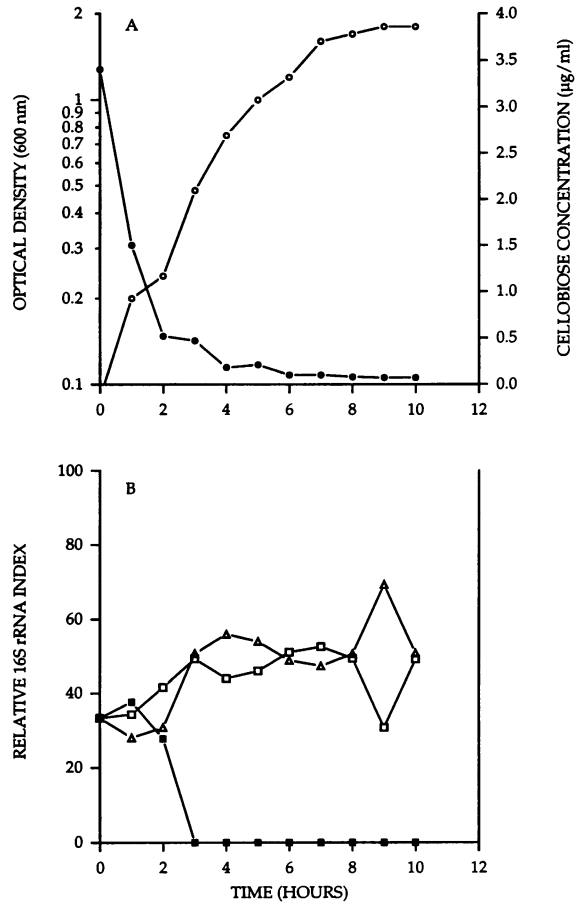

FIG. 8. Bacterial growth, substrate disappearance, and relative 16S rRNA indexes (percent) of a cellobiose-grown triculture of *R. albus* 8, *R. flavefaciens* FD-1, and *F. succinogenes* S85. (A) Bacterial growth (○) measured by observing the optical density at 600 nm, and substrate disappearance (●) measured as the total sugar concentration. (B) Slot blot quantitation of *R. albus* 8 (□), *R. flavefaciens* FD-1 (■), and *F. succinogenes* S85 (△) with *R. albus* probe RAL196, *R. flavefaciens* probe RFL196, and *F. succinogenes* probe SUB1.

advantage would appear to be in the coupling of cellobiose uptake to energy-generating processes. *F. succinogenes* S85 metabolizes cellobiose by using a cellobiase (15), whereas *R. flavefaciens* FD-1 metabolizes cellobiose via a cellobiose phosphorylase (17). The phosphorolytic cleavage of cellobiose, especially if linked to cellobiose uptake, represents a considerable increase in ATP formed per mole of hexose fermented and thus an overall increase in growth efficiency for this bacterium (28). This increase in growth efficiency would theoretically give *R. flavefaciens* FD-1 an advantage over *F. succinogenes* S85 when cellobiose was the sole carbon and energy source. This may result in a lower M_e, allowing more energy to be diverted to growth-related processes, thus conferring a competitive advantage.

The observation that *R. flavefaciens* FD-1 does not compete well with *R. albus* 8 when cellobiose is the growth substrate does not appear to be due to a nutritional advantage inherent in *R. albus* 8, because the inhibition occurred while cellobiose was still available. The inhibition was therefore not due to competition for nutrients but was due to production of a bacteriocin-like substance. Further, this inhibition appears specific for *R. albus* 8 toward *R. flavefaciens* FD-1, because

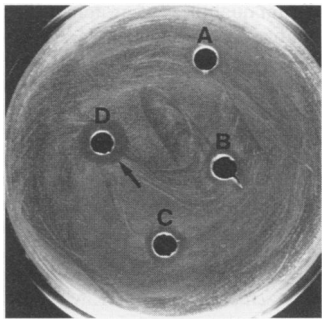

FIG. 9. Production of a bacteriocin-like activity by *R. albus* 8 against *R. flavefaciens* FD-1. Agar well diffusion assay showing the activity of the partially purified bacteriocin-like substance (60% ammonium sulfate precipitation) from *R. albus* 8 against the indicator *R. flavefaciens* FD-1. Wells: A, uninoculated media; B and C, ammonium sulfate precipitation of *R. albus* 8 culture supernatant after dialysis and filtration; D, ammonium sulfate precipitation of *R. albus* 8 culture supernatant after dialysis without filtration.

other experiments show that the bacteriocin-like substance was not inhibitory to *F. succinogenes* S85 or other strains of *R. albus* (data not shown). This characteristic, a narrow spectrum of action toward a genus or species, is one of the criteria which defines a bacteriocin (34). Further, although many bacteriocins or bacteriocin-like substances have been identified in a number of gram-positive bacteria, especially lactic acid bacteria (19, 26, 27), this is the first demonstration of the production of these substances by ruminal bacteria. Again, these results would not be detected by conventional techniques but are clear when bacterial population dynamics are measured by using relative 16S rRNA levels.

The results indicate that the approaches reported in this study represent a model system for studying bacterial interactions of ruminal microorganisms in defined mixed cultures with specific substrates. This work has also demonstrated the complexity of interactions that occur between the ruminal fibrolytic bacteria. Future work will focus on the competition between these ruminal fibrolytic bacteria when insoluble substrates for growth are used (see the accompanying paper [24a]).

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REFERENCES

1. **Amann, R. I., B. Binder, S. W. Chisholm, R. Olsen, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
3. **Amann, R. I., N. Springer, W. Ludwig, K.-H. Schleifer, and N. Peterson.** 1991. Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. Nature (London) **351:**161–164.
4. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:** 614–623.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for quanti-

tation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
7. **Bryant, M. P.** 1972. Commentary on Hungate technique for culture of anaerobic bacteria. Fed. Proc. **32**:1808–1813.
8. **Daba, H., S. Pandian, J. F. Gosselin, R. E. Simard, J. Huang, and C. Lacroix.** 1991. Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. Appl. Environ. Microbiol. **57**:3450–3455.
9. **Davey, G. P., and B. C. Richardson.** 1981. Purification and some properties of Diplococein from *Streptococcus cremoris*. Appl. Environ. Microbiol. **41**:84–89.
10. **Dehority, B. A.** 1993. Microbial ecology of cell wall fermentation, p. 425–453. *In* H. J. Jung, R. Hatfield, and P. Weimer (ed.), Forage cell wall structure and digestibility. American Society for Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc., Madison, Wis.
11. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360–1363.
12. **Devereux, R., M. D. Kane, J. Winfrey, and D. A. Stahl.** 1992. Genus and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst. Appl. Microbiol. **15**:601–609.
13. **Dubois, M., K. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorometric method for determination of sugars and related substances. Anal. Biochem. **28**:350–353.
14. **Geis, A., J. Singh, and M. Teuber.** 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. **43**:205–211.
15. **Groleau, D., and C. W. Forsberg.** 1981. Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. Can. J. Microbiol. **27**:517–530.
16. **Hahn, D., M. J. C. Starrenburg, and A. D. L. Akkermans.** 1990. Oligonucleotide probes that hybridize with rRNA as a tool to study *Frankia* strains in root nodules. Appl. Environ. Microbiol. **56**:1342–1346.
17. **Helaszek, C. T., and B. A. White.** 1991. Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. Appl. Environ. Microbiol. **57**:64–68.
18. **King, W. S., S. Raposa, J. Warshaw, A. Johnson, D. Harbert, and J. Klinger.** 1989. A new colorimetric nucleic acid hybridization assay for *Listeria* in foods. Int. J. Food Microbiol. **8**:225–232.
19. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70**:337–349.
20. **Lathe, R.** 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. J. Mol. Biol. **183**:1–12.
21. **Maas, L. K., and T. L. Glass.** 1991. Cellobiose uptake by the cellulolytic ruminal anaerobe *Fibrobacter* (*Bacteroides*) *succinogenes*. Can. J. Microbiol. **37**:141–147.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **McSweeney, C. S., R. I. Mackie, A. A. Odenyo, and D. A. Stahl.** 1993. Development of an oligonucleotide probe and its application for detection and quantitation of the ruminal bacterium *Synergistes jonesii* in a mixed-population chemostat. Appl. Environ. Microbiol. **59**:1607–1612.
24. **Odenyo, A. A., R. I. Mackie, G. C. Fahey, Jr., and B. A. White.** 1991. Degradation of wheat straw and alkaline hydrogen peroxide-treated wheat straw by *Ruminococcus albus* 8 and *Ruminococcus flavefaciens* FD-1. J. Anim. Sci. **69**:819–126.

24a. **Odenyo, A. A., R. I. Mackie, D. A. Stahl, and B. A. White.** 1994. The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: pure-culture studies with cellulose and alkaline peroxide-treated wheat straw. Appl. Environ. Microbiol. **60**:3697–3703.

24b. **Odenyo, A. A., R. I. Mackie, and B. A. White.** 1992. The use of 16S rRNA targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria, p. 396. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.

25. **Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W. M. Kuan, T. J. Macke, Y. Q. Xing, and C. R. Woese.** 1992. The ribosomal database project. Nucleic Acids Res. **20**:2199–2200.
26. **Piard, J. C., P. M. Muriana, M. J. Desmazeaud, and T. R. Klaenhammer.** 1992. Purification and partial characterization of lactacin 481, a lanthionine-containing bacteriocin produced by *Lactobacillus lactis* subsp. *lactis* CNRZ 481. Appl. Environ. Microbiol. **58**:279–284.
27. **Rammelsberg, M., E. Muller, and F. Radler.** 1990. Caseicin 80: purification and characterization of a new bacteriocin from *Lactobacillus casei*. Arch. Microbiol. **154**:249–252.
28. **Rasmussen, M. A., R. B. Hespell, B. A. White, and R. J. Bothast.** 1988. Inhibitory effects of methylcellulose on cellulose degradation by *Ruminococcus flavefaciens*. Appl. Environ. Microbiol. **54**:890–897.
29. **Stahl, D. A.** 1986. Evolution, ecology, and diagnostics: unity in variety. Bio/Technology **4**:623–628.
30. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, England.
31. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079–1084.
32. **Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace.** 1984. Analysis of hydothermal vent-associated symbionts by ribosomal RNA sequence. Science **224**:409–411.
33. **Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace.** 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. **49**:1379–1384.

33a. **Stahl, D. A., et al.** Unpublished data.

34. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-negative bacteria. Bacteriol. Rev. **40**:722–756.
35. **Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson.** 1990. Use of oligonucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol. **56**:2858–2865.
36. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.